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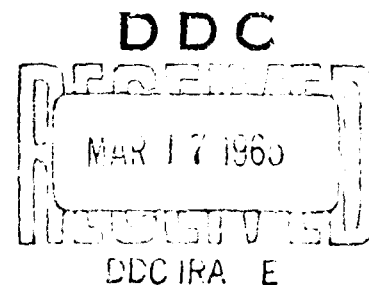
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A MECHANISM OF ACTION
OF STAPHYLOCOCCAL ENTEROTOXIN POISONING

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Although food poisoning has been a significant medical problem since biblical times, Albrecht von Haller (1) apparently conducted the first scientific study in the early 1800's. In 1894 Van de Velde (2,3) published the first reference to staphylococcal food poisoning and Denys (4) incriminated a yellow micrococcus in an outbreak. There followed many case reports and epidemiologic studies of outbreaks of food poisoning with several excellent clinical reviews (3,5,6,7,8,9).

Since the mid-thirties, basic investigations have employed classical pharmacologic and physiologic preparations such as isolated intestinal systems, muscle with and without attached nerve, uterine strips, perfused heart, frog skin, and many others. Although crude preparations of toxin possessed some physiologic action, as the material was purified, activity was lost. Bayliss (10), using young and adult cats and a variety of surgical techniques, concluded that the action of the enterotoxin was primarily on the peripheral sensory structures. In opposition to this concept, Sugiyama and his colleagues (11) stated that a critical factor in the action of the enterotoxin is its effect on the chemoreceptor trigger zone located in the area postrema. Not only have they ablated this section of the brain, preventing the emetic reaction, but they have also shown a synergism between the emetic effect of dihydroergotamine, which is said to act through the chemoreceptor trigger zone, and that of staphylococcal enterotoxin. In addition, Sugiyama, et al (12) have shown that perphenazine and reserpine have a definite anti-emetic effect. While the reserpine must be used as a pre-treatment, the perphenazine was effective even when given 45 minutes after an intravenous dose of the enterotoxin. Inasmuch as perphenazine is said to be anti-emetic for those substances acting upon the chemoreceptor trigger zone, these workers believe that this observation provides additional support for the conclusions stated above. Orlov (13) also concluded that there was little or no action on the gastrointestinal tract but on a

specific part of the CNS.

With the isolation of a reproducibly pure preparation of staphylococcal enterotoxin, type BII (14), a detailed study of its distribution, metabolism, and attendant physiologic changes was begun.

METHODS

Labeling of Toxin: In order to carry out the distribution studies, the toxin has been labeled with Iodine-131. The procedure is essentially as follows: 2 ml of a 1% solution of the enterotoxin in pH 7.4 phosphate buffer is incubated with a mixture containing 0.4 ml of 127 mg I₂ in 0.2M KI and 1 ml of carrier-free I¹³¹ obtained from a commercial source* averaging 1.0 mc per ml of radioactivity. The mixture is allowed to react for 30 minutes when it is separated from the unreacted iodide I¹³¹ by passing it successively through 2 separate columns of amberlite IRA-400 resin. This procedure yielded a product that had about 20 atoms of I¹³¹ per molecule of toxin and less than 5% unbound I¹³¹. When tested in dogs, the toxicity was identical with the starting material. This labeled product was used in the distribution studies to be reported below. Human serum albumin was labeled in the same way at the same concentration and used in control studies.

Disappearance Times: One ml blood samples were taken from monkeys at 10, 20, 30, 45 and 60 minutes and at 2, 3, 5, 8, 12 and 24 hours post-challenge in order to follow the rate of loss of the labeled toxin and albumin from the blood.

Blood Binding Studies: The in vitro distribution of staphylococcal enterotoxin was studied by collecting 10 ml of blood from normal monkeys. Amounts varying from 20 to 1,000 µg of I¹³¹ labeled toxin were added to various samples of blood. The mixture was incubated either at room temperature for 1 hour or at 37°C for 30 minutes. Following incubation, 1 ml of the mixture was assayed for radioactivity. The whole blood was then separated into platelet, leukocyte, plasma, and erythrocyte fractions. Formed elements were washed with normal monkey serum. Radioactivity of each fraction was determined and the per cent of activity in all fractions computed. One ml samples of plasma were treated with an equal volume of 20% TCA. The supernate was separated and radioactivity determined. The precipitate was washed 3 times with 5% TCA and the radioactivity of each wash, as well as the precipitate, was determined.

In vivo binding of the toxin within the blood was studied by challenging 5 monkeys with varying dose levels of labeled toxin (20 to 200 µg per kg). Ten ml samples of blood were taken at 15 minutes and 3 hours post-challenge. Following determination of

*Na Radio-Iodide (Oriodide) Abbott Laboratories, Oak Ridge, Tenn.

radioactivity in whole blood, the buffy coat was removed and radioactivity of the platelets, leukocytes, erythrocytes, and plasma was determined in the same manner as described above. It should be pointed out that total blood volume is a limiting factor in all studies in which blood must be removed from monkeys.

Distribution and Physiologic Studies: The distribution of and physiologic response to I^{131} labeled staphylococcal enterotoxin was followed in several species of animals. Additional animals of the same species were treated with a similar quantity of I^{131} labeled human albumin to serve as controls. The various species used and the number of animals in each group are summarized in Table I. Food and water were available ad libitum throughout the course of the experiment for all animals.

TABLE I. NUMBER OF ANIMALS USED IN SERIAL SACRIFICE STUDIES

<u>SPECIES</u>	<u>TYPE</u>	<u>TOXIN</u>	<u>ALBUMIN</u>
Rats	Sprague-Dawley	30	10
Rabbits	Mixed	30	9
Monkeys	<u>Macaca mulatta</u>	28	10
Previously challenged monkeys	<u>Macaca mulatta</u>	9	

The rats and rabbits were treated per os with Lugol's solution 24 hours prior to challenge to block the uptake of the I^{131} by the thyroid. Rats were challenged with 20 μ g/kg or with 40 μ g/kg of toxin or albumin via the penal vein. Three rats from each of the toxin groups were sacrificed at 1, 3, 6, 12 and 24 hours post-challenge. One each of the albumin controls were killed at the same time. Eighteen of the rats receiving toxin were placed in individual metabolic cages to collect urine. Rats were euthanized with chloroform and the remainder of the animals with "Lethol"* given either iv or directly into the heart.

Rabbits were similarly divided and challenged at the 2 dosage levels via an ear vein. Three animals of each toxin dose level and 1 control (40 μ g/kg) were killed at 1 hour. Subsequently, of each dose level, 4 of the principals and 1 of the controls were killed at 6, 12 and 24 hours. No urine was collected from rabbits.

Three groups of monkeys were used, those which had no previous experience with the enterotoxin, hereafter referred to as clean monkeys; those which had been previously exposed, hereafter termed re-challenge monkeys; and controls. In all studies reported here, monkeys were placed in specially designed metabolic chairs and a

*26% pentobarbital Na and 13% amobarbital Na in a base of isopropanol and polyethylene glycol, Corn State Laboratories, Omaha, Nebraska.

polyethylene cannula was placed in a peripheral vein into the posterior vena cava to facilitate blood sampling. Urine collections were made from 3 monkeys in each group. All monkeys were given either a 20 µg/kg dose of toxin or albumin iv. Two of the principals were sacrificed at 1 and 3 hours post-challenge. Subsequently, 3 of the clean monkeys, as well as 3 of the re-challenge monkeys, were killed at 6, 12 and 24 hours. Two of the controls were killed at each time interval.

Tissues from all animals were removed immediately following sacrifice. Whole organs were weighed to the nearest 0.05 gm and placed in plastic tubes for assay of radioactivity. In cases where the organs were large, a representative sample approximating 1 gram was taken, weighed and counted. Calculations were made on a per gram, as well as a per organ, basis. The following tissues were taken: liver, lung, kidney, spleen, heart, brain cortex, pancreas, adrenals, pituitary, thyroid (from monkeys only), large intestine, small intestine, stomach, and skeletal muscle. In addition, urine, feces, stomach contents, and blood samples were obtained from all animals, as well as bile from the gall bladder of monkeys.

All samples were counted in a well-type crystal scintillation counter to an error of less than 5%.

To further examine the organ distribution and the nature of the material as to whether or not it was protein bound, a second group of 15 clean monkeys were handled as described previously. However, these monkeys were challenged with 100 µg/kg of toxin, iv. Three of these animals were killed at 1, 3, 6, 12 and 24 hours. The same organs and tissue were removed, weighed and radioactivity ascertained as in other monkeys. In addition, homogenates were made of lung, liver and spleen and the concentration of radioactivity was determined. A volume of homogenate was treated with an equal volume of 20% TCA. The radioactivity of both the precipitate and supernate was assayed. From these results the per cent of the radioactivity still protein bound was determined.

Studies of Body Spaces: Body water compartments were determined by using tritiated water as a measure of total body water, and radiosulfate or inulin as measures of extracellular water in nephrectomized monkeys.

Inulin was assayed using Roe's resorcinol method as modified by Schreiner (15). Tritiated water and radiosulfate were assayed using a liquid scintillation β-particle spectrometer (16). Because previous work with staphylococcal enterotoxin challenged monkeys indicated renal impairment (17), the variable of renal excretion was eliminated by bilaterally nephrectomizing each monkey. Six or 7 hours following surgery, the inulin and tritiated water or radiosulfate and tritiated water were injected intravenously. Five

blood samples for pre-challenge values were taken during a 3-hour period, which started 8 to 9 hours after injection of the tracers. Immediately after the last pre-challenge blood samples were drawn, the monkeys were challenged iv with the toxin.

The monkeys in which inulin was used received 25 $\mu\text{g}/\text{kg}$ of toxin and the monkeys in which the radiosulfate was used received 200 $\mu\text{g}/\text{kg}$. At 0.5, 1, 2, 3 and 4 hours post-challenge, blood samples were collected using Sequester-Sol* as an anticoagulant and a protein-free filtrate (Folin-Wu) was made from the plasma.

In other non-nephrectomized monkeys, RISA** and hematocrits were used as indicators of vascular water shift. For each monkey 1.5 μC of RISA was injected into a superficial arm vein. Control blood samples were collected at 24, 25, 27, 28, 29, 45, 46, 47 and 48 hours following RISA injection to insure that the metabolic phase of the RISA distribution curve had been reached. Radioactivity per ml of serum was measured in a well-type crystal scintillation counter. Micro-hematocrits were done in duplicate at the 45, 46, 47 and 48 hour sampling times.

Immediately following the 48-hour control sample, each monkey was challenged intravenously with 25 $\mu\text{g}/\text{kg}$ of the staphylococcal enterotoxin. Blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 hours post-challenge. RISA content and hematocrits were measured as described above.

RESULTS

Symptomatology: Rats appeared to be resistant to the effects of the toxin at the dose administered. Rabbits exhibited a variable response, but generally symptoms were mild and short-lived, and, as with the rats, none died during the 24-hour period of study. Nearly 100% of the clean monkeys showed the usual clinical signs of illness after the first hour. These included vomiting, diarrhea, anorexia, pyrexia, pale mucous membranes, and mild to severe depression leading to death. None died in the first 24 hours of the study. However, many animals used in other concurrent studies have died within 24 hours following this dose. Only 2 of 9 monkeys, both having the lowest anti-enterotoxin titer of the group, showed any symptoms upon re-challenge during the 24-hour period of observation. Both were moderately depressed and vomiting was noted in one.

Disappearance Times: Table II summarizes the disappearance rates of the injected macromolecules. The initial half-life of I^{131} labeled toxin in the blood of 20 clean monkeys ranged from 10 to 45

*Dipotassium ethylenediamine tetra-acetate, Cambridge Chemical Products, Inc., Dearborn, Michigan.

**Radio-iodinated (I^{131}) serum albumin, Abbott Laboratories, Oak Ridge, Tennessee.

minutes, while that of 6 re-challenged monkeys varied from 10 to 60 minutes and that of albumin under these conditions ranged from 200 to 400 minutes. However, the continued rate of loss of the albumin and toxin are similar.

TABLE II. RATE OF LOSS OF LABELED STAPHYLOCOCCAL ENTEROTOXIN AND ALBUMIN FROM THE BLOOD OF MONKEYS*

	CLEAN ENTEROTOXIN <u>T 1/2 IN MINUTES**</u>	RE-CHALLENGE ENTEROTOXIN <u>T 1/2 IN MINUTES</u>	ALBUMIN <u>T 1/2 IN MINUTES</u>
Average	18	28	275
Range	10-45	10-60	200-400

*Allowing 10 minutes for mixing.

**T 1/2 = time required for the concentration to decrease by a factor of 2.

Blood Binding Studies: The in vitro and in vivo distribution of the labeled enterotoxin within the blood has been studied. In vitro studies have shown that 15 to 20% of the label is bound to the buffy coat and the remainder to the albumin fraction of the plasma. All radioactivity is removed from the erythrocytes with 3 washes of serum. However, results from in vivo studies indicate that at 15 minutes following administration of toxin less than 1% of the dose is combined to the buffy coat, and by 3 hours there is no detectable buffy coat activity remaining. Following washes by serum, there is no activity on the erythrocytes at any time. At 15 minutes post-challenge, 90% of the label is protein bound within the plasma. Later, at 3 hours, as much as 70% of the amount within the plasma is protein free.

Tissue Distribution Studies: The results from studies of urine demonstrated considerable differences between rats (resistant) and monkeys and between clean and re-challenged monkeys in their ability to clear the I¹³¹. The results of urinary I¹³¹ clearance are indicated in Figure 3. It appears that those animals having the greatest resistance to the effects of the toxin excrete the greatest amount of the label in the urine. This observation has also been noted in clean monkeys, i.e., those showing minimal clinical symptoms voided larger amounts of I¹³¹ in the urine. It was further found that the accumulation of I¹³¹ in the thyroid of the re-challenged monkeys was 4 times greater than the uptake by the thyroid of clean monkeys (17.3% to 4.5%). This and the urine studies indicate that the I¹³¹ was being freed from the protein faster in previously challenged or more resistant animals and is probably an indication of a faster rate of metabolism of the toxin molecule. Limited data indicates that the liberated I¹³¹ is primarily in the form of inorganic iodide rather than as iodinated amino acids as is the case with the iodinated albumin.

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The results of the tissue studies have shown minimal variation in the organ concentration from animal to animal within a species. Figures 4 and 5 illustrate the change in organ concentration with time after challenge. It is apparent that, except as noted below, the labeled material disappears from the tissue in a manner similar to that for albumin. There was no appreciable difference in the distribution of the enterotoxin in rats or rabbits at the higher dose level of either albumin or toxin.

There appears to be a selective concentration of the enterotoxin in the lungs of all species studied as compared with the albumin. This concentration remains elevated for a considerable length of time in clean monkeys at a time when the blood level is falling. Furthermore, this activity is largely protein bound. Radioactivity has been noted in the lungs of monkeys dying from 4 to 10 days following challenge, whereas activity in other organs was minimal or undetectable. A major difference seen among rats and rabbits (resistant animals) as compared to monkeys in the distribution of the toxin was this continuing high concentration in the lungs of the monkeys. The anomalous situation of the sustained high level of activity in the lungs of the re-challenged, resistant monkeys may be explained by the not unreasonable assumption that the labeled toxin combined with its antibody has been fixed by the phagocytes in the lungs.

It should be noted further that the toxin concentration rises in the heart and kidneys for the first 6 to 12 hours and then falls off in the same manner as albumin.

Body Space Studies: No significant differences between the pre-challenge and post-challenge total body water compartment (TBW) could be detected with T20. Likewise, no changes in the extracellular fluid compartment (ECF) could be shown with either the inulin or the radiosulfate. These findings indicate that the intracellular water (ICW) (TBW-ECF) remains unchanged. An increase in RISA concentration occurred within 1 hour post-challenge as shown in Figure 1, and can best be explained by a loss of vascular water. This conclusion is supported by the rise in hematocrit, as shown in Figure 2.

TABLE III. INCREASE IN LUNG WEIGHT FOLLOWING ENTEROTOXIN CHALLENGE

<u>NO. OF MONKEYS</u> <u>CONTROL/TOXIN</u>		<u>HOURS POST-</u> <u>CHALLENGE</u> <u>SACRIFICE</u>	<u>RATIO OF LUNG/HEART</u> <u>CONTROL/TOXIN</u>		<u>DIFFERENCE</u> <u>SIGNIFICANT</u> <u>AT % LEVEL</u>
10	5	1	1.43	1.43	-
10	5	3	1.43	1.98	10
10	6	6	1.43	1.94	10
10	6	12	1.43	2.17	5
10	6	24	1.43	2.53	10

This rise occurs after a steady declining hematocrit due to the removal of red blood cells by sampling. Since there is no change in the extracellular or intracellular water, this loss of vascular water is best explained by a shift into the interstitial space. A very interesting observation was the increase in weight of the lungs of the clean monkeys at sacrifice as compared to the control monkeys as seen in Table III. This change might well reflect the shift of fluids seen in body water compartment studies.

DISCUSSION

From the above results a possible mechanism of action of staphylococcal enterotoxin may be postulated. In vitro studies have shown 15 to 20% of the labeled toxin combined with the buffy coat. However, less than 1% was found combined with the buffy coat from in vivo studies 15 minutes following iv administration of the toxic material. It is logical to assume that the material is being bound by the leukocytes which in turn are being cleared rapidly from the circulation by some organ. It has been shown that when leukocytes are altered in some manner by a variety of procedures, they are rapidly removed from the circulation by the lungs (18, 19). Furthermore, it has been shown that the disappearance of the toxin from the circulating blood is considerably faster than that of an equal amount of albumin. Therefore, it may be postulated that the lung is removing the leukocyte-bound toxin from the circulation. This theory is supported by (a) the high concentration of the label found within the lung at sacrifice and (b) by an increase in the number of circulating young and immature leukocytes, suggesting rapid replacement of mature cells. Other workers have also reported a high concentration of I¹³¹ within the lungs following challenge with a labeled exotoxin (20). Further, this high concentration within the lungs continues in non-resistant animals, whereas it falls rapidly in resistant animals (rats and rabbits). Animals which are resistant because of previous experience with the toxin also have continuing high levels of label in the lungs. As pointed out above, this may be explained by postulating that the antigen-antibody complex is being fixed by the phagocytes of the lung. A major part of the radioactivity found within the lung of clean monkeys following challenge is protein bound, suggesting the material is the same as that administered.

Excretion studies have demonstrated that the label is eliminated in the urine much more rapidly from resistant than from non-resistant animals. This label has been shown to be almost entirely inorganic iodide suggesting that it is a direct measure of the ability of the animal to metabolize the toxin. Further evidence of this is the large amount of label being concentrated in the thyroid of previously challenged, resistant, monkeys. Furthermore, it appears that even among non-resistant animals, those which are better able to eliminate the label in the urine are more apt to survive. It thus appears that toxin metabolism, as measured by the appearance of Iodide-131, is associated with decreased effectiveness. Since the

lungs of clean and re-challenged monkeys contain about the same amount of radioactivity, and since the re-challenged monkeys are eliminating much more of the label, it would seem that the lung may not be the major organ involved in breakdown of the toxin. This observation is supported by the large amount of radioactivity found in the livers of re-challenged monkeys at the 6-hour sacrifice and the rapid fall-off of radioactivity in subsequent sacrifices, suggesting the liver as a major organ of metabolism.

Results of water distribution within the various body compartments clearly show a loss of fluid from intravascular space, but not from the entire body. As pointed out above, the water is lost into the interstitial space of the extracellular fluid. Preliminary evidence, summarized in Table III, indicates that there is an accumulation of fluid in the lung. Furthermore, a significant histopathologic lesion seen in monkeys is an accumulation of fluid in the interstitial spaces of the lung.* Scattered alveoli may contain fluid but frank pulmonary edema is seldom seen. Likewise, several of the symptoms seen in animals suggest some type of interference with the exchange of gases, for example, dyspnea and cyanosis.

As yet the exact mechanisms involved in death from staphylococcal enterotoxin is merely speculation, but it appears to be closely related to a reaction within the lungs. Thus, illness and subsequent death may be due to interference with gas diffusion in the lungs resulting from an accumulation of fluids in the interstitial spaces. However, additional work must be and is being carried out to determine the details of this mechanism.

SUMMARY

These studies provide no information concerning the possible role of the CNS in the pathogenesis of acute staphylococcal toxin reactions, nor to the cause of vomiting and diarrhea. They do suggest that (a) the toxin is bound initially to WBC's and albumin, (b) that toxin-bound WBC's are trapped in the lung, (c) that the lungs develop increased weight due to interstitial fluid accumulation which occurs coincident with loss of vascular fluid, but with unchanged total body water, ICW and ECF, and (d) that re-challenged and resistant animals handle toxin in a different fashion, evidenced by an early increased accumulation of I^{131} label in the liver and an increased clearance of I^{131} free of protein by the kidney and thyroid.

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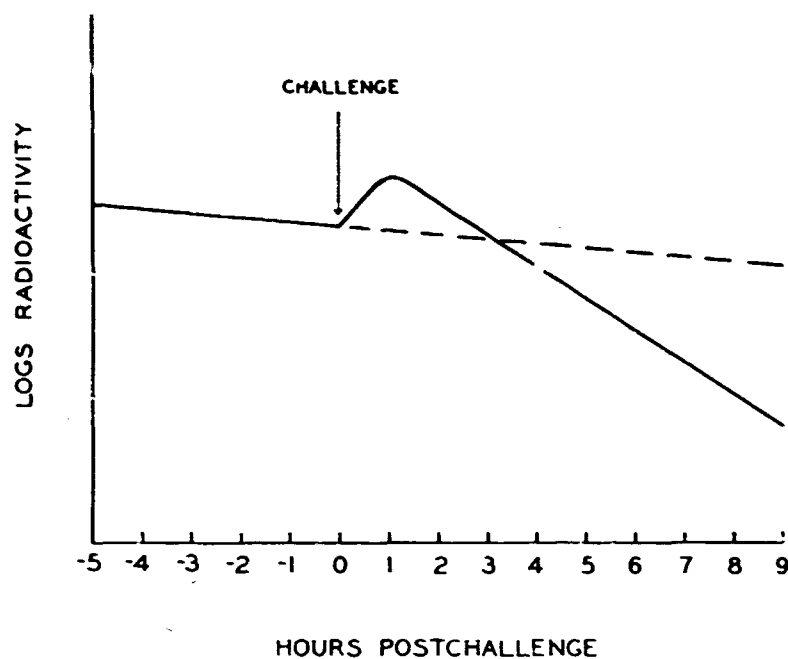


Figure 1: Changes in RISA Distribution

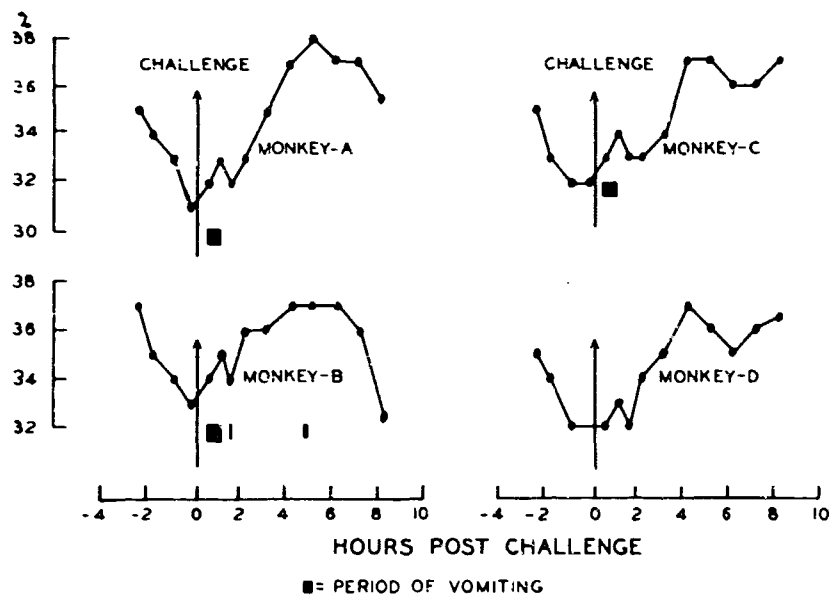


Figure 2: Changes in Hematocrits

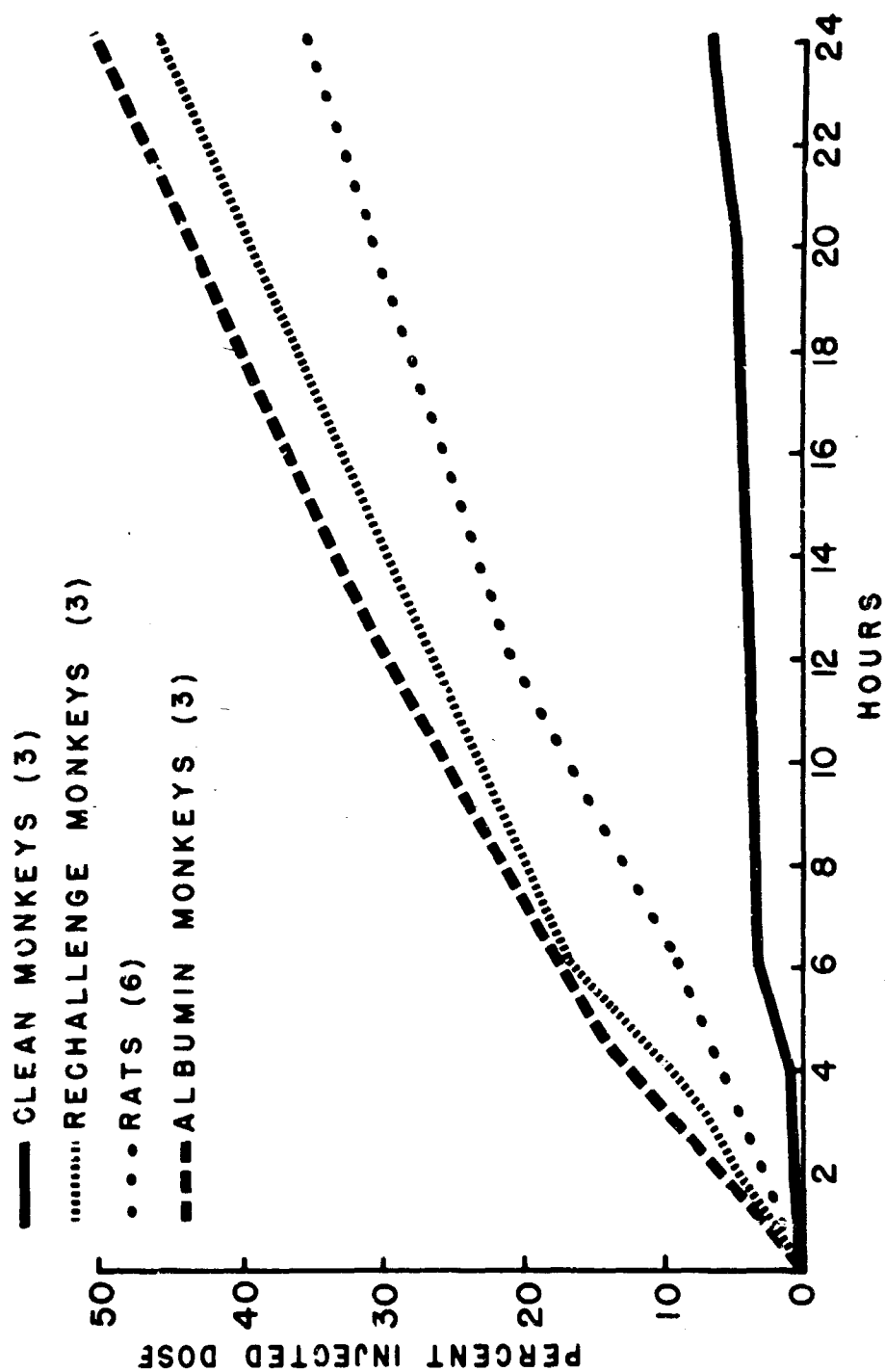


Figure 3: Urinary I^{131} Excretion

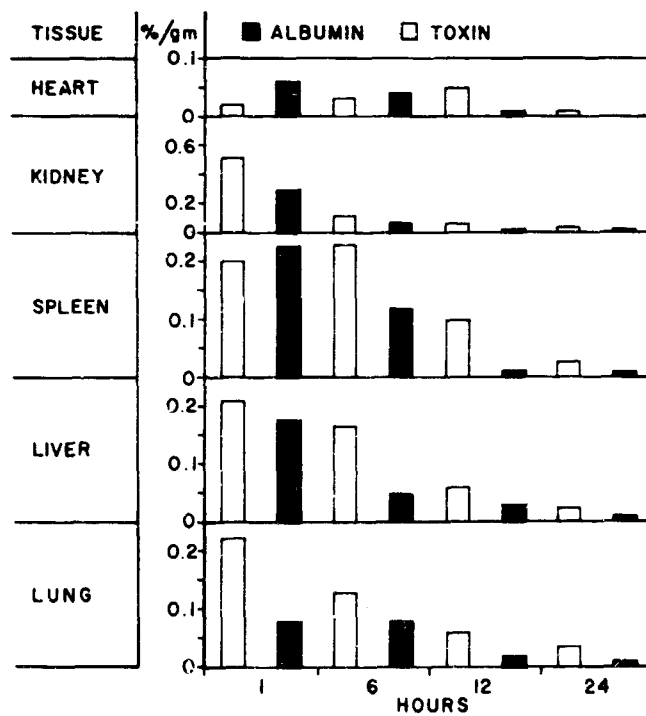
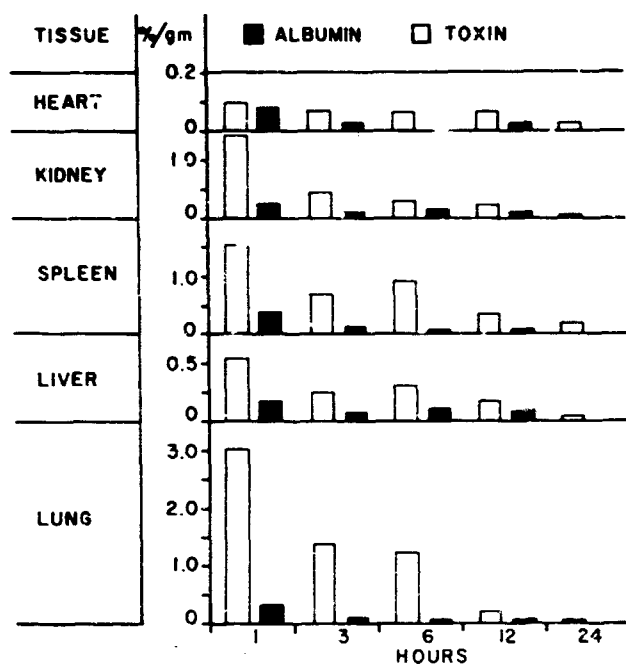


Figure 4: Distribution of I^{131} in Tissues (wet) Relative to Time
a. Rats
b. Rabbits

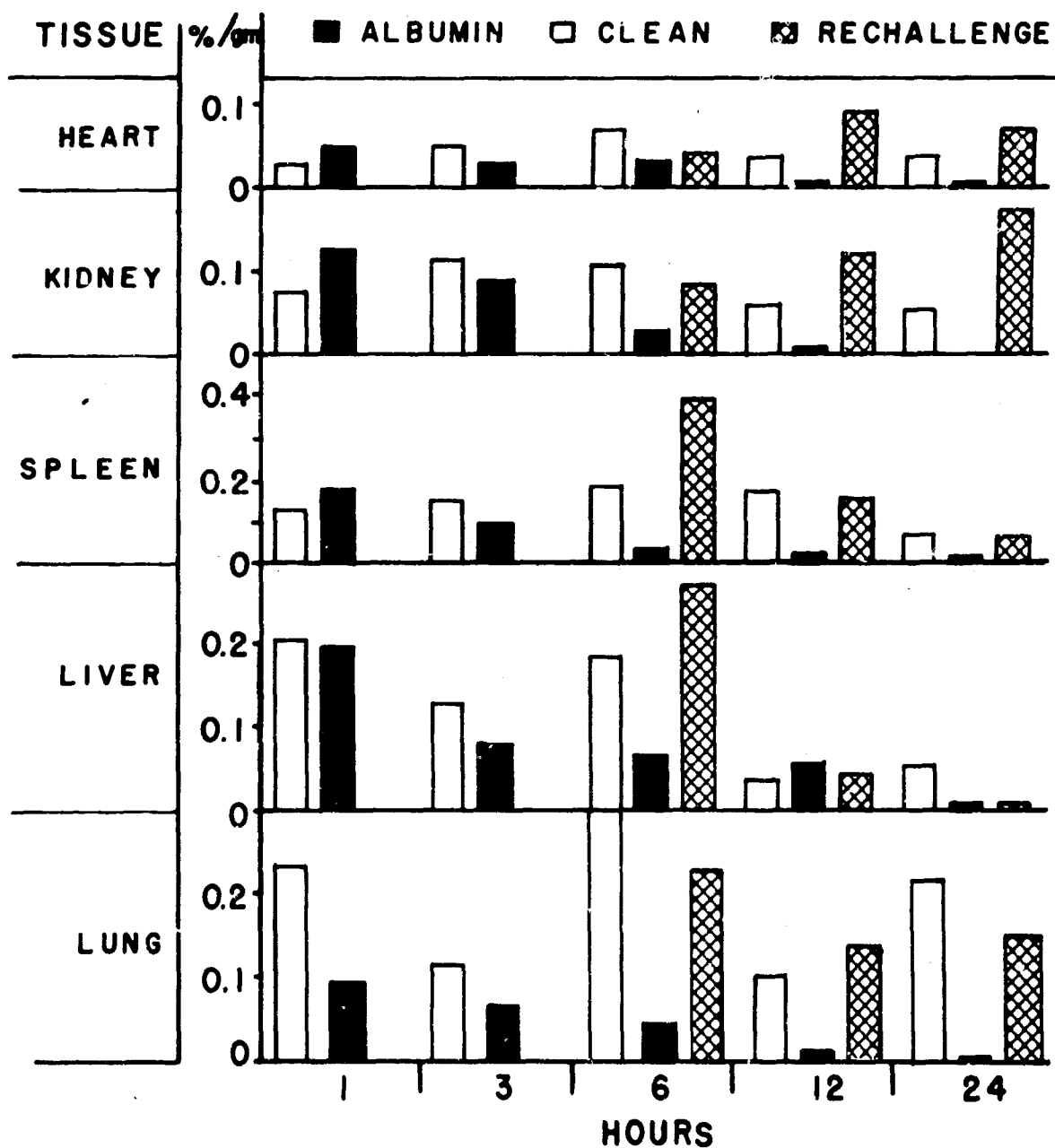


Figure 5: Distribution of I^{131} in Monkey Tissues (wet) Relative to Time